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MYCORRHIZAL SYMBIONTS OF THE TERRESTRIAL ORCHID *CYPRIPEDIUM FASCICULATUM*

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ABSTRACT. Three populations of *Cypripedium fasciculatum,* the clustered lady's slipper orchid, and its associated mycorrhizal fungi were the subject of our study in southern Oregon. These orchids maintain a mycorrhizal relationship into maturity. Although most fungi-forming orchid mycorrhizas are saprophytes or necrotrophic parasites, some non-photosynthetic, achlorophyllous orchids are mycoheterotrophs. These orchids derive all their carbon through association with fungi that obtain carbon via ectomycorrhizal relationships with trees. By analysis of fungal DNA with PCR-RFLP and DNA sequencing, we found that *C. fasciculatum* associates with several fungal species, at least one of which belongs to the family Russulaceae, including common ectomycorrhizal fungi in coniferous forests. The same fungi also were found in rhizomes of the non-photosynthetic orchid *Corallorhiza.* Stable isotope analysis of orchid and non-orchid tissues indicates that digestion of fungal biomass in root cells supplies *C. fasciculatum* with substantial proportions of both carbon and nitrogen. Although *C. fasciculatum* is green and presumably photosynthetic under favorable conditions, our results indicate that the species also has the ability to parasitize fungi as an intermediate between the trophic patterns of non-photosynthetic, mycoheterotrophic orchids and photosynthetic non-orchids. These results, which elucidate the ecological connections of *C. fasciculatum,* have implications for managing and conserving the species and its accompanying fungi.

Key words: *Cypripedium,* mycorrhiza, Russulaceae, mycoheterotrophy, stable isotope

INTRODUCTION

Cypripedium fasciculatum S. Watson, the clustered lady's slipper orchid, is a rare terrestrial orchid endemic to western North America. Most populations occur in mature coniferous forests with 70–90% canopy cover. In Oregon, *C. fasciculatum* occurs in Douglas-fir (*Pseudotsuga menziesii*) forests (Latham & Hibbs 2001) at least 70 years old (R. Knecht unpubl. data).

Cypripedium fasciculatum, like most terrestrial orchids, requires contact with an appropriate fungus for germination and maintains fungal infections in its roots into maturity (Beyrle et al. 1995, Cribb 1997, Smith & Read 1997). Blue-staining fungi with internal coils (pelotons) and fungi with dark septate hyphae on the root surface have been found with *C. fasciculatum* roots (E. Cazares unpubl. data). The identity and function of these mycorrhizal interactions, however, are for the most part undescribed (Seevers & Lang 1998).

Among eight *Cypripedium fasciculatum* plants, 43 fungal taxa were identified by molecular analysis of DNA isolated from fungi cultured out of roots (F. Camacho unpubl. data). These included fungi not usually associated with orchids, such as *Fusarium, Nodulisporum, Penicillium,* and *Sordaria,* as well as the members of the form genus *Rhizoctonia* spp. Orchid mycorrhizas originally assigned to the form genus *Rhizoctonia* have been re-classified in recognition of their diverse evolutionary lineages. Much of the past research on orchid mycorrhizas relied on culturing fungi from pieces of orchid roots; but improved DNA technology demonstrates that culturing techniques fail to identify some important mycorrhizal fungi (Allen et al. 2003).

Orchids associate with a diverse array of fungi from many trophic niches (TABLE 1). Many orchid-associated fungi are saprotrophic and obtain carbon by breaking down organic matter. Several necrotrophic fungi, including the forest pathogen *Armillaria mellea,* associate with orchids (Currah & Zelmer 1992). Shelf fungi such as *Fomes* sp. form mycorrhizas with some orchids (Warcup 1981). A number of orchids, including achlorophyllous, a non-photosynthetic species, associate with ectomycorrhizal fungi (Warcup 1991).

Orchids have a special relationship with mycorrhizal fungi: carbon flows from fungus to plant, while no clear benefit for the fungus has been demonstrated (Hadley & Purves 1974, Purves & Hadley 1976, Alexander & Hadley 1985). Fungal hyphae enter orchid root cortical cells and form coiled clumps of hyphae called pelotons. The pelotons eventually degrade, and

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TABLE 1. Trophic status of fungi known to form mycorrhizal relationships with orchids.

Telomorph fungus	Fungal synonymy	Fungal trophic status	Orchid associates	Source
Armillaria		Parasitic	*Gastrodia elata*	Lan et al. 1994
Ceratobasidium	*Ypsilonidium, Thanatephorus, Ceratorhiza*	Saprotrophic, parasitic	*Goodyera, Platanthera,* others	Currah et al. 1990
Erythromyces	*Hymenochaete*	Saprotrophic	*Galeola, Erythrorchis*	Umata 1995
Fomes		Saprotrophic (wood decaying)	*Galeola*	Burgeff 1959
Marasmius		Saprotrophic	*Didymoplexis*	Burgeff 1959
Moniliopsis		Unknown	*Platanthera*	Currah et al. 1990, Zelmer et al. 1996
Mycena		Saprotrophic	*Cymbidium, Gastrodia*	Fan et al. 1996
Phellinus		Saprotrophic (wood decaying)	*Galeola*	Umata 1995
Russulaceae		Ectomycorrhizal	*Corallorhiza*	Taylor & Bruns 1999
Sebacina	*Serendipita*	Saprotrophic, ectomycorrhizal	*Acianthus*	Roberts 1999
Sistotrema		Saprotrophic	*Piperia, Platanthera*	Currah et al. 1990
Thanatephorus		Parasitic	*Calypso*	Currah 1987
Thelephoraceae		Ectomycorrhizal	*Cephalanthera, Corallorhiza*	Taylor & Bruns 1997
Tulasnella	*Epulorhiza, Rhizoctonia repens*	Saprotrophic, possibly ectomycorrhizal	*Dendrobium,* others	Warcup & Talbot 1980, Warcup 1981

the orchid absorbs the contents of the fungal cells (Arditti 1992).

Achlorophyllous orchids, such as *Corallorhiza* and *Cephalanthera,* are mycoheterotrophic deriving all their carbon from ectomycorrhizal fungi, which in turn receive carbon from nearby trees (Zelmer & Currah 1995, Warcup 1991). Photosynthetic orchids also can obtain significant percentages of carbon and nitrogen from digested fungal biomass (Gebauer & Meyer 2003). Stable isotope analysis was employed as a means of tracking the flow of carbon and nitrogen in the ecosystem. Hobbie and Colpaert (2003) suggested that ^{15}N signatures could reflect the amount of carbon allocated by a photosynthesizing tree to fungal symbionts. Fungal enzymatic processes generally discriminate in favor of ^{15}N (Emmerton et al. 2001). Ectomycorrhizal fungi function like fine roots to deliver nitrogen to plants, except for orchids, which absorb fungal biomass. Thus orchids should be more enriched in ^{15}N than ectomycorrhizal plants, with the degree of enrichment reflecting the importance of mycoheterotrophic sources of nutrition.

The purpose of this study was to investigate the relationship between *Cypripedium fasciculatum* and its mycorrhizal associates in an effort to discover (1) the identity of fungal symbionts, (2) the role those symbionts play in the nutrition of *C. fasciculatum,* and (3) the implications for understanding the ecology and distribution of this plant.

METHODS

Cypripedium fasciculatum S. Watson plants were excavated from three sites in Jackson County, Oregon. The Alexander Gulch site (42°9′32″N, 123°9′27″W) in the Applegate River watershed at 1042 m elevation had a canopy closure of 60% in a mature forest of Douglas-fir (*Pseudotsuga menziesii)* mixed with madrone (*Arbutus menziesii),* with an understory of *Symphoricarpos alba, Ceanothus* sp., and *Berberis nervosa.* The Gold Hill site (42°28′27″N, 123°1′22″W) at 786 m elevation had canopy closure of 80% with Douglas-fir and madrone dominant. The Butte Falls site (2°34′55″N, 122°36′31″W) at 615 m elevation had 90% canopy closure in mature Douglas-fir and white fir forest with maple *(Acer macrophylum* and *A. circinatum)* understory.

Hand-cut root sections were stained by soaking in chlorazol black for 2–3 days and then examined with a compound microscope.

Roots of *Calypso bulbosa* (L.) Oakes, *Goodyera oblongifolia* Raf, *Piperia* sp., and *Corallorhiza* sp., occurring in the vicinity of *Cypripedium fasciculatum,* were collected to determine if they have the same mycorrhizal symbionts as *C. fasciculatum.* Fungal fruiting bodies and ectomycorrhizal root tips from Douglas-fir trees in the area also were collected for comparison to orchid mycorrhizas.

One-mm slices of roots were taken from each plant, and fungal DNA then was extracted fol-

lowing Gardes and Bruns (1993). The ITS1, 5.8s rDNA, and ITS2, along with partial 18s and 28s regions, were amplified using fungal specific primers ITS1-F and ITS4 (Bruns et al. 1998, Gardes & Bruns 1993). DNA was amplified by polymerase chain reaction (PCR) in a thermocycler with 1 cycle at 94°C for 2 min., followed by 30 cycles of 94°C for 1 min., followed by 51°C for 1 min., and 72°C for 2 min., followed by one additional cycle of 72°C for 5 min. and holding at 4°C. The resulting sequences have been submitted to the public database GenBank.

The fungus *Tulasnella* is known to associate with orchids (TABLE 1), and in some cases to facilitate mycoheterotrophy (Bidartondo et al. 2003). Since *Tulasnella* often is not amplified by ITS1-F/ITS4 primers, samples also were amplified with primers ITS1 and ITS-4 that are designed to target *Tulasnella* (White et al. 1990, Taylor 1997). PCR products were cut with *hinf*I and *taq*I enzymes (Gardes & Bruns 1993), and restriction fragment length polymorphisms (RFLPs) were separated in 4% acrylamide gels. After staining with ethidium bromide, RFLP bands were photographed under UV light and analyzed with ONE-Dscan software. The RFLP patterns were compared to an existing database of local southern Oregon mycorrhizal fungi and to the RFLP patterns of mushrooms gathered in the vicinity of the orchid populations.

PCR products were cleaned using Microcon columns and prepared for sequencing using ¼ reactions of BigDye Terminator Ready Reaction mix from Applied Biosystems DNA sequencing kit. Samples then were sequenced in both directions with an ABI Prism 310 Genetic Analyzer. Sequence data was cleaned and analyzed using Chromas and Seqed, and Clustal software, and compared to GenBank, using a BLAST search (Altschul et al. 1997) to identify similar sequences.

In some samples where more than one species of fungus was present in orchid root samples, multiple species were separated by cloning with an Invitrogen TOPO TA cloning® kit. In the cloning reaction, 3.5 μL of PCR product, 1.0 μL salt solution, and 0.8 μL of TOPO vector were incubated at room temperature for 30 min. The chemically competent *Escherichia coli* cells provided in the kit were transformed following the manufacturer's instructions, with the exception of heat-shocking, which was done for 1 min. Thirty-five μL of X-Gal was added to *E. coli,* and samples were spread on LB Media plates containing 100 μg/ml ampicillin. Colonies were screened and the white colonies placed in 100 μL of liquid LB Media containing 100 μg/ml ampicillin. These colonies were incubated for 18–24 h at 37°C. After incubation, 24 colonies from each sample were amplified and run on acrylamide gels.

For stable isotope analysis of carbon and nitrogen, roots and shoots of orchids, adjacent plants, and fungal sporocarps were dried and ground. Analysis was done on an automated $^{15}N/^{13}C$ analyzer-mass spectrometer (Europa Scientific, Crewe, UK) at the stable isotope facility at the University of California at Davis.

Stable isotope data were analyzed using the isotope-mixing model described by Gebauer and Meyer (2003):

$$(\varepsilon_{MHO\text{-}R} = \delta x_{MHO} - \delta x_R):$$

$$\%x_{dF} = \delta x_{AO} - \delta x_R / \varepsilon_{MHO\text{-}R} = 100\%$$

where MHO is mycoheterotrophic orchid, AO is autotrophic orchid, R is reference plant, %dF is the percentage of fungally derived nutrient, and X is carbon or nitrogen isotope ratio. Samples from the mycoheterotrophic orchid *Corallorhiza* sp. were used as a standard for 100% carbon and nitrogen derived through mycoheterotrophy. Ectomycorrhizal tree tissue samples were used as the standard for zero carbon and nitrogen derived by mycoheterotrophy.

RESULTS

Morphology

Root cortical cells of *Cypripedium fasciculatum* collected in May 2003 contained pelotons in a degraded condition. Other orchids, such as *Goodyera oblongifolia* collected in April 2003 and *Corallorhiza* sp. collected in July 2003, had active infections of intact hyphae.

Fungi Extracted from Roots

From DNA extracted from *Cypripedium fasciculatum* roots, 13 RFLP patterns representing distinct taxonomic entities were identified (TABLE 2). Six RFLP patterns were obtained from plants from Alexander Gulch (AG), four more from Butte Falls (BF), and four from Gold Hill (GH), one of which was also found at AG. Three samples from AG were sequenced and identified based on BLAST similarity as *Russula, Lactarius,* and *Suillus.* One cloned sample from GH was sequenced and identified as *Tulasnella* based on BLAST similarity (TABLE 2).

Some *Cypripedium fasciculatum* RFLP patterns matched RFLP patterns of fungi extracted from other orchid roots or ectomycorrhizal root tips (TABLE 3). *Cypripedium fasciculatum* RFLP type 8 matched *Corallorhiza* sp. root endophytes and ectomycorrhizal root tips identified as *Russula* by BLAST similarity. RFLP type 6 matched

TABLE 2. RFLP patterns of fungal DNA extracted from roots of *Cypripedium fasciculatum* from Alexander Gulch (AG), Butte Falls (BF), and Gold Hill (GH).

Type	*Hinf*1a	*Hinf*1b	*Hinf*1c	*Hinf*1d	*Hinf*1e	*Taq*a	*Taq*b	*Taq*c	*Taq*d	*Taq*e	*Taq*f	*Taq*g	Site	BLAST ID
1	177	115				266	164						AG, GH	
2	184	170	120			251	190	157					AG	
3	356	234	171	157	148	314	229	174	152				AG	*Suillus*
4	363	323				301							GH	
5	368	355				381	296	266	189	174	123	110	AG	*Lactarius*
6	373	348				293	236	160					BF	
7	377	244	179			445	309	238	80				AG	
8	404	356				413	323	295					AG	*Russula*
9	437	323				438	274	156					GH	*Tulasnella*
10	447	333				475	380	286					BF	
11	450	290	189			328	283	167	149				GH	
12	464	337				287	280						BF	
13	483	339				364	288	174	151				BF	

an ectomycorrhizal root tip identified as *Tomentella* by BLAST search.

RFLP type 14, extracted from roots of the orchid *Goodyera oblongifolia,* was identified as *Ceratobasidium* by BLAST. This RFLP type was not found in any *Cypripedium fasciculatum* roots, in ectomycorrhizas, or in other orchids (TABLE 3).

The fungus obtained from roots of the orchid *Piperia* sp. was identified as *Epulorhiza* with BLAST. Although *Epulorhiza* is the anamorph form of *Tulasnella* (Currah & Zelmer 1992), the *Tulasnella* targeted combination of ITS1/ITS4-Tul did not amplify this sample. The DNA sequence of this type was nearly identical to that of RFLP type 9, identified by sequence as *Tulasnella,* from roots of *Cypripedium fasciculatum* from GH (TABLE 2).

Stable Isotopes

Corallorhiza sp. tissues yielded significantly elevated ^{15}N values compared to all other plants, with a $\delta^{15}N$ of 14.5 0/00 (FIGURE 1). The green orchids *Cypripedium fasciculatum* and *Goodyera oblongifolia* were enriched in ^{15}N relative to ectomycorrhizal plants, which had ^{15}N values similar to atmospheric nitrogen (FIGURE 1). Values of $\delta^{13}C$ did not demonstrate as distinct a pattern as ^{15}N, although $\delta^{13}C$ values of *Corallorhiza* sp. were similar to nearby *Russula* sp. (FIGURE 1). The combined $\delta^{15}N$ and $\delta^{13}C$ values of samples revealed similarity between sample types. Ectomycorrhizal plant tissues tended toward more negative $\delta^{15}N$ and $\delta^{13}C$ values, while *Corallorhiza* sp. displayed the highest $\delta^{15}N$ and $\delta^{13}C$ values, and mushrooms, *C. fasciculatum,* and *G. oblongifolia* were intermediate (FIGURE 1). Using the twin isotope-mixing model, *C. fasciculatum* from AG obtained 10–18% of its nitrogen and 42–46% of its carbon from fungal sources. *Goodyera oblongifolia* received only 10–12% of its carbon from fungal sources.

DISCUSSION

All of the BLAST-identified fungi found in the roots of *Cypripedium fasciculatum* were potentially ectomycorrhizal with dominant conifers near the orchids. Fungi from the Russulaceae and Thelephoraceae were the most common ectomycorrhizas in pinaceous forests in California (Bruns et al. 2001). *Tulasnella* also can form ectomycorrhizas with coniferous trees (Bidartondo et al. 2003).

Furthermore, three of the fungal groups found

TABLE 3. RFLP patterns of DNA from roots of *Goodyera oblongifolia* (GOOB), *Piperia* sp. (PISP), *Corallorhiza* sp. (COSP), and ectomycorrhizas (ECTO) collected at Alexander Gulch (AG), Butte Falls (BF), and Gold Hill (GH).

Type	*Hinf*1a	*Hinf*1b	*Hinf*1c	*Taq*a	*Taq*b	*Taq*c	*Taq*d	*Taq*e	Host	Site	BLAST ID
6	367	367		289	231				ECTO	BF	*Tomentella*
8	426	370		425	329	295			ECTO	BF	*Russula*
8	439	377		430	336	300			COSP	AG	*Russula*
9	412	364	307	314	291	280	188	164	PISP	GH	*Tulasnella*
14	370	277		365	300				GOOB	AG	*Ceratobasidium*

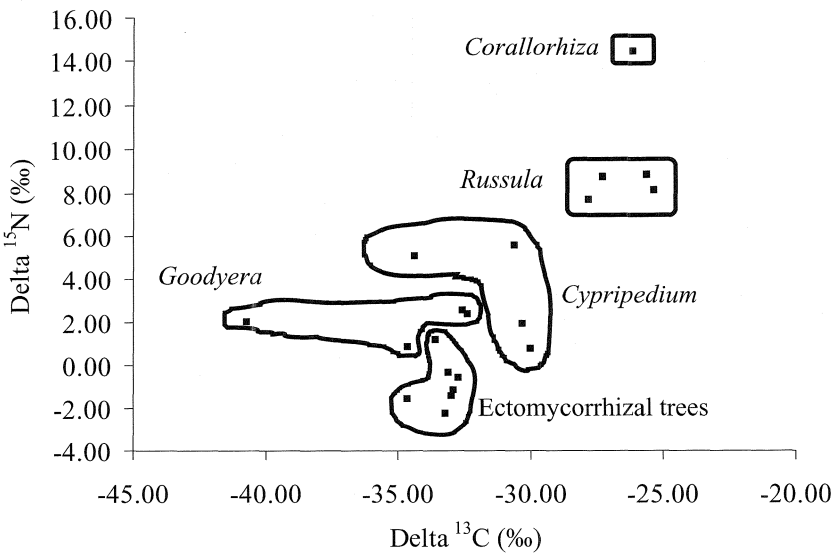

FIGURE 1. Natural abundance of stable isotope ratios, δ^{15}N and δ^{13}C, of the orchids *Cypripedium fasciculatum* and *Goodyera oblongifolia,* of the mushroom *Russula* sp., and of photosynthetic, ectomycorrhizal plants including Douglas-fir. Clusters of stable isotope signatures show *C. fasciculatum* intermediate between non-photosynthetic *Corallorhiza* sp. and photosynthetic ectomycorrhizal trees.

in *Cypripedium fasciculatum* roots support mycoheterotrophy in other plants. Fungi from the Russulaceae were found in species of the achlorophyllous *Corallorhiza* (Taylor & Bruns 1999). Others from the Thelophoraceae were found in the mycoheterotrophic orchids *Cephalanthera* and *Corallorhiza* (Taylor & Bruns 1997). *Tulasnella* supported the achlorophyllous liverwort *Cryptothallus* (Bidartondo et al. 2003). Other green orchids co-occuring with *C. fasciculatum* associate with saprotrophic or parasitic fungi. *Goodyera oblongifolia* contained the saprotroph *Ceratobasidium* in its roots. *Calypso bulbosa,* which grew near *C. fasciculatum* at all sites, was associated with the parasitic *Thanatephorus* in Canada (Currah 1987). In contrast to these other green orchids, the fungi in *C. fasciculatum* belonged to distinctly ectomycorrhizal trophic niches. Furthermore, the fungus found in rhizomes of *Corallorhiza* matched one of the *C. fasciculatum* associates. The three fungi in *C. fasciculatum* roots are consistent with the hypothesis that this orchid is partially mycoheterotrophic, receiving a significant portion of its carbon in the form of photosynthate from nearby trees via fungi.

Stable isotope data support the partially mycoheterotrophic nature of *Cypripedium fasciculatum.* Ectomycorrhizal fungi have access to large amounts of carbon in the form of photosynthate from dominant overstory trees (Smith & Read 1997), which would facilitate the 42–46% fungally derived carbon found in the tissues of *C. fasciculatum.* Access to this carbon would provide an important advantage to a green orchid in the low light conditions found at all three sites.

The heterogeneous distribution of *Cypripedium fasciculatum* could be partially explained by the presence or absence of fungi capable of supporting partial mycoheterotrophy. Understanding the trophic status of the symbionts also should assist management and conservation of this rare orchid, since the presence of large photosynthetic trees appears to be required to support *C. fasciculatum.*

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